MicroRNA-21 deficiency promotes the early Th1 immune responses and resistance towards visceral leishmaniasis

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Microbiology in the undergraduate colleges of The Ohio State University

by

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Abstract

MicroRNA-21 (miR-21) is known to inhibit IL-12 expression and impair the development of a Th1 immune response necessary for control of *Leishmania* infection. It has been recently shown that *Leishmania* infection induces miR-21 expression in dendritic cells and macrophages and inhibition of miR-21 restores IL-12 expression. Since miR-21 is known to be expressed due to inflammatory stimuli in a wide range of hematopoietic cells, we investigated the role of miR-21 in shaping innate and adaptive immune responses during *Leishmania donovani* infection. We found that miR-21 expression was significantly elevated in dendritic cells, macrophages, inflammatory monocytes, PMNs, and in the spleen, liver tissues following *Leishmania donovani* infection, concomitant with an increased expression of IL-6 and STAT3. BMDCs from miR-21 null mutant mice showed an increased production of IL-12 and decreased production of IL-10. Further, upon *L. donovani* infection, miR-21 KO mice exhibited significantly higher numbers of IFN-γ and TNF-α producing CD4+ and CD8+ T cells in both livers and spleens, that was accompanied by increased production of Th1-associated IFN-y, TNF-α, and Nitric Oxide (NO) from the splenocytes. Finally, miR-21KO mice showed significantly higher numbers of developing and mature hepatic granulomas resulting in reduced parasitic loads in the livers and spleens compared to similarly infected wild type mice. These observations suggest that induction of miR-21 results in exacerbation of this disease and also confirms the direct suppressive role of miR-21 on IL-12 and IL-12 induced IFN-γ associated Th1 immune reactions. In conclusion, miR-21 promotes susceptibility to *L. donovani* infection by complimenting IL-6, STAT-3 signaling, and inhibiting the development of Th1 immune responses and can therefore be used as a potential target for the resolution of VL disease.

Introduction

Micro-RNAs (miRNAs) are a class of small single-stranded non-coding RNA molecules that serve to regulate gene expression in eukaryotes. They bind to mRNA at the transcriptional and post-transcriptional levels to repress translation or degrade the target mRNA (1). Interaction of miRNAs with their targets has been broadly studied and has been shown to mediate an array of crucial cellular and pathophysiological processes such as tumorigenesis and regulation of the host's immune response including inflammation, cell differentiation, and immunity development (2). Moreover, the effector functions of macrophages and T cells can depend on miRNA regulation (3). Micro-RNA-21 (miR-21) is a highly expressed miRNA in multiple mammalian cell types and was first investigated as an oncogene whose up-regulation functions to promote numerous cancers (4). Its up-regulation has been associated with inflammatory diseases such as asthma and psoriasis, as well as bacterial and viral infections (5). miR-21 expression has also been shown to increase during the activation of various immune cells including T cells, dendritic cells (DCs), macrophages, monocytes, and neutrophils in different disease states (5).

Leishmaniasis is a neglected tropical disease caused by infection with obligate protozoan parasites and is found in about 90 countries but most prevalently in tropic and subtropics regions (6). Due to the lack of resources needed to treat this disease in many impoverished areas, leishmaniasis has become the second most common fatal parasitic infection next to malaria, affecting approximately 12 million people worldwide (7). The most severe form is visceral leishmaniasis (VL), which affects the host's internal organs such as the spleen, liver, and bone marrow (6). VL possesses a mortality rate of almost 100% and results primarily from infection with the species *L. donovani* (8). Despite the high mortality resulting from this disease, there

continues to be a lack of effective treatment for VL, which highlights the need for further scientific research.

The innate and adaptive immune responses elicited from the host play a major role in directing the fate of *Leishmania* infection. The complexity of immune responses, different host interactions with various *Leishmania* species show an effect on vaccine, diagnostic, and drug development strategies (9). T cells, DCs, and macrophages are known to play a vital role in cellular immunity (10) and their coordinated effector functions result in the pro- and anti-inflammatory immune responses induced by *L. donovani* infection (8). It is known that the pro-inflammatory Th1 response is crucial for host resistance against VL that is driven by the inflammatory cytokines IL-12 and IFN-y, which promote phagocyte activation (11) and death of intracellular parasites (3). In contrast, chronic VL infection is characterized by a switch towards the alternate anti-inflammatory Th2 response, whose mediators effectively neutralize anti-parasitic inflammatory responses (12), which leads to lower phagocytic activity and higher disease susceptibility.

The potential role of miRNA in leishmaniasis has been highlighted by previous miRNA profiling studies during the *Leishmania* infection (13-15). Pro-inflammatory T helper (Th1) and anti-inflammatory Th2 cells normally exist in a controlled balance, however, previous studies have described miR-21 as a key factor in switching host's T cell immunity towards Th2 (3, 4, 16). Following activation of immune cells, increased miR-21 expression has been shown to skew this balance to promote an anti-inflammatory environment and reduce Th1 immune responses through targeting the production of certain inflammatory cytokines by T cells and phagocytes (11). Furthermore, previous studies have shown that targeted ablation of miR21 in antigen-presenting DCs leads to increased production of Th1 cytokine IL-12, meanwhile, miR-21 ablation in CD4+ T cells leads to increased production of Th1 mediator IFN-y and decreased Th2 mediator IL-4

production, portraying the importance of miR-21 in regulating polarized immuno-inflammatory responses (17). It was also shown that miR-21 deficiency promotes macrophage polarization towards M1-like phenotype and immunity against various disease conditions (18). Therefore, miR-21 remains a target of interest in *Leishmania* infection, whose resolution relies heavily on T cell immunity for clearance of the parasite. miR-21 expression has also been correlated with TGF-β signaling pathways especially SMAD7 and TRAF6 in *L. donovani* infection (13). Regulation of IL-12 in live attenuated *Leishmania* parasite-infected DCs by miR-21 highlighted its role in vaccine-induced protective immunity (19). Further, previous studies have identified increased expression of miR-21 in *L. infantum* infected tissues of canine VL infection (11, 15). Following activation of immune cells in a study using *L. Infantum* model, increased miR-21 expression was shown to skew this balance to promote an anti-inflammatory environment and reduce Th1 immune responses through targeting the production of certain inflammatory cytokines by T cells and phagocytes (11). Thus, it is suggested that miR-21 acts to induce a Th1 suppressed state during *Leishmania* infection. However, the mechanism by which miR-21 mediates T cell immunity and the inflammatory response is still unclear. In the present study, we investigated the role of miR-21 in *L. donovani* induced pathogenicity by infecting WT and miR-21 gene-deficient (miR-21KO) mice and evaluating the immune-modulatory responses and the course of infection. Our results show that *L. donovani* infection increases miR-21 expression and that miR-21 contributes to the progression of *L. donovani* infection by inhibiting IL-12 induced IFN-γ-Th1 immune responses and the mechanism behind this regulation to be through the IL-6 and STAT3 signaling axis.

Methods

Animals

C57bl/6 WT and miR-21KO mice were purchased from The Jackson Laboratory (BL/6 Stock No: 000664 and miR-21KO Stock No: 016856) Bar Harbor, ME, USA. Mice were maintained and bred at The Ohio State University Laboratory Animal Resources (OSU-ULAR) facilities. All experiments were performed using age-matched 7-8 week old female mice under an approved protocol (2010A0048-R3) by OSU-Institutional Animal Care and Use Committee (OSU-IACUC).

L. donovani infection and analysis of parasitic loads

LV82 strain of *L. donovani* amastigotes was obtained from the spleens of previously infected golden strain hamsters. Infected spleens were mashed, and amastigotes were counted. All experimental mice were infected with 10^7 amastigotes via tail vein injection. Infected mice were euthanized at respective time intervals post-infection (POI), spleens and livers were harvested to make a smear impression on glass slides. Smears were fixed with methanol, air-dried, and stained with Giemsa stain (Sigma-Aldrich, St. Louis, MO, USA). Parasitic burdens were calculated by enumerating the number of amastigotes per 1000 nucleated cells and represented as Leishman-Donavan Units (LDU) using the following formula: LDU= number of amastigotes/1000 nucleated cells x tissue weight in grams.

Histopathology analysis

Livers were harvested from all experimental mice at respective time intervals, fixed with formalin and sectioned at 5microns, and stained with hematoxylin and eosin. The numbers and

types of granulomas were determined by a pathologist and scored as follows: (i) developing granuloma (with an initial influx of lymphocytes and monocytes and presence of amastigotes, (ii) mature/functional granuloma, (iii) parasite-free granuloma (granuloma devoid of amastigotes. Granuloma counts were representative of the average of 8-10 individual mice from one of the 3 identical studies.

miR-21 expression in infected tissues and phagocytic cells

C57BL/6 WT mice were infected with *L. donovani* LV82 strain amastigotes, after 24 hours of POI, spleens, and livers were harvested. RNA was isolated from the infected and uninfected tissues to analyze miR-21 expression by RT-qPCR as described below.

C57BL/6 WT mice were infected with transgenic *L. donovani* LV82 amastigotes expressing the red fluorescent protein DsRed2 for 24hrs. Spleens were harvested at 24 hours POI, and single-cell suspensions were prepared from the tissues. Red blood cells (RBCs) were lysed by ACK lysis buffer. Cells were stained with the respective cocktail mix containing antibodies (anti-mouse-BV510-CD11b, PECy7-F4/80, PacificBlue-CD11c, AF700-MHCII, FITC-Ly6G and APC-Ly6C, purchased from BioLegend, San Diego, CA, USA) for isolation of parasitized/not parasitized macrophages (CD11b+Ly6C^{medium}Ly6G-ve), dendritic cells (DCs/CD11C+MHCII), polymorphonuclear neutrophils (PMNs/CD11b+Ly6G+) and inflammatory monocytes (iMOS/CD11b+Ly6C^{hi}Ly6G-ve) by using BD FACS ARIA III sorter (BD Biosciences, San Jose, CA). Total RNA was isolated from the cells by using a miRNeasy mini kit (Qiagen, Germantown, MD), cDNA was prepared by TaqMan Advanced miRNA cDNA synthesis kit (Thermo Fisher Scientific, Grand Island, NY). miR-21 gene expression was determined by TaqMan advanced

miRNA assay (catalog No: A25576, Assay ID: 477975_miR, Thermo Fisher Scientific). The data were analyzed by CFX manager software.

Analysis of cytokine and nitric oxide production

Mice were euthanized at respective time intervals, spleens were harvested to make single-cell suspensions. RBCs were lysed by ACK lysis buffer and cells were plated at a concentration of $0.5x10^6$ cells/well in 96 well tissue culture plates in RPMI1640 supplemented with 10%FBS and 1% penicillin and streptomycin. Cells were stimulated with 20µg/ml *L. donovani* antigen (LdAg, prepared by freeze-thawing *L. donovani* promastigotes) for 72 hours, culture supernatants were collected and measured the cytokine production. Purified and Biotin anti-mouse IFN-γ, TNF-α, IL-4, IL-10, IL-12 Primary and secondary antibodies of cytokines were purchased from BioLegend (San Diego, CA) and all the standards were purchased from BD Biosciences (San Jose, CA). Production of nitric oxide from culture supernatants was measured by Greiss assay.

In vitro bone marrow-derived dendritic cell assay

Bone marrow cells were isolated from WT and miR-21KO mice, RBCs were lysed and plated at $5x10^6$ cells/flask in sterile tissue culture flasks along with RPMI 1640 medium supplemented with 10% FBS, 1% Penicillin streptomycin (Life Technologies), and 20ng/ml of GMCSF (Peprotech) for 6-7 days. BMDCs were collected by gently scraping the cells, plated in 24 well plates treated with/without 1mg/ml LPS (Sigma Aldrich) for 24hrs. LPS treated and untreated BMDCs were infected with *L. donovani* promastigotes (BMDCs: parasites in the ratio of 1:10) for 24 hours. Culture supernatants were collected and determined IL-12 and IL-10 cytokine production.

Flow cytometry analysis

Single-cell suspensions were prepared from spleens and livers of mice at respective time points. RBCs were lysed by ACK lysis buffer, cells were stained with respective cocktail mixes. For the determination of cytokine-producing T cell populations by intracellular flow cytometry, cells were stimulated with an activation cocktail (purchased from BioLegend) for 4 hours, surface stained with anti-mouse BV421-CD3, PE-Cy5-CD4, AF-700-CD8, PE-NK1.1, permeabilized and stained with anti-mouse APC-IFN-γ, FITC-TNF-α, and APC-Cy7-IL-10 (All antibodies purchased from BioLegend). For analyzing myeloid cell populations such as M1 macrophages (F4/80+, CD11C+), M2 macrophages (F4/80+, CD206+), iMOS (CD11b+Ly6C^{hi}), and PMNs (CD11b+Ly6G+), samples were stained with anti-mouse BV510-CD11b, PacificBlue-CD11C, PECy7-F4/80, Percp/Cy5.5-CD206, APC-Ly6C, and FITC-Ly6G. Cells were acquired by BD LSRFortessa (BD Biosciences) and data were analyzed by FlowJo software V10 (Tree Star Inc, Ashland, OR).

Statistical analysis

Unpaired Student *t*-tests were used to determine the statistical significance of differences between experimental and control groups by using GraphPad software. A *P* value of <0.05 was considered significant.

Results

1. *Leishmania donovani* induces miR21 expression *in vivo*.

High expression of miR-21 is considered as indicative of the "active" state of many immune cells and not only regulates numerous cell functions but also plays a key regulator role in orchestrating the balance and transition between pro- and anti-inflammatory responses. Since early host-parasite interactions determine the infection outcome, miR-21 could play a critical role in shaping host immune responses following *L. donovani* infection. To investigate whether the miR-21 expression is effected by the *L. donovani* infection in various cell types that potentially harbor *Leishmania* parasites, we infected WT BL/6 mice with transgenic DsRed2 *L. donovani* LV82 parasites, after 24hrs of infection we isolated parasitized macrophages, DCs, PMNs, and iMOS from the spleen (Fig 1A) and quantified the miR-21 5p expression. We found that miR-21 expression was significantly increased (1.5 fold) in *L. donovani* infected cells compared to respective un-infected control cells isolated from WT naïve mice (Fig 1B). We also found that *L. donovani* infection-induced miR-21 expression significantly in the spleen (1.6 fold) and liver (1.2 fold) (Fig 1C).

Since the interleukin-6 (IL-6) regulates microRNA-21 (miR-21), functioning via activation of signal transducers and activators of transcription 3 (STAT3) in various neoplastic cells (20-22), we wanted to explore the IL-6/STAT-3 axis in the regulation of miR-21 in the context of *L. donovani* infection. Recent studies have shown that activation of STAT3 is associated with VL infection and inhibition of STAT3 led to decreased parasitic loads (23). Elevated levels of IL-6 in both experimental and clinical VL have also been reported, and IL-6 deficient mice control the VL infections faster than WT mice (24-27). To investigate the role of IL-6/STAT3 signaling in

inducing miR-21 up-regulation, we analyzed the gene transcripts of *IL-6*, and *STAT3*, in *L. donovani* infected spleen and liver and compared with naïve counterparts. Similarly, we measured IL-12 in these tissues since miR-21 has been shown to regulate IL-12 in previous studies.

Our results showed that *Leishmania* infected livers and spleens expressed 4-fold increased *IL-6* and 6 fold increased *STAT3* transcripts compared to the uninfected controls indicating that *Leishmania* induced miR-21 expression is associated with IL-6 and STAT3 up-regulation (Figs 1D & E). Although no difference was observed in *IL-12* expression in the livers (Fig 1D), *L. donovani* infection significantly reduced *IL-12* expression in the spleens indicating the direct inhibitory role of miR-21, which expression was induced by *L. donovani* (Fig 1E).

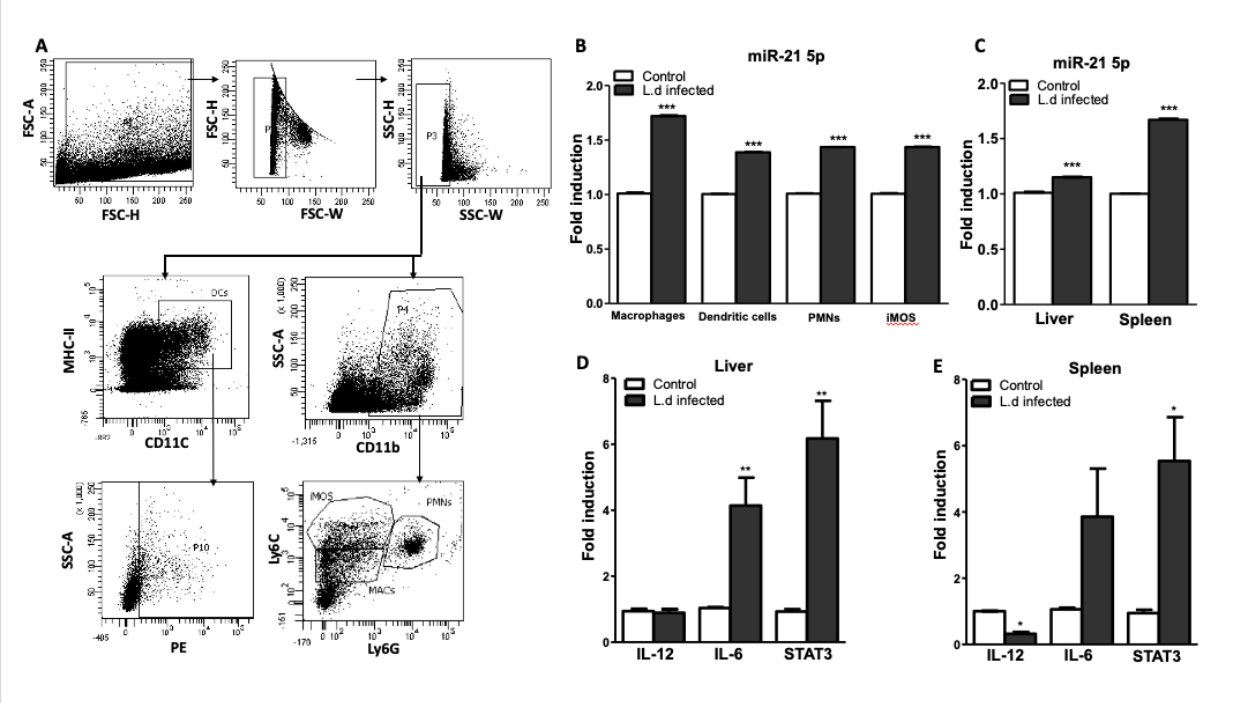

Figure 1. Expression of miR-21 in visceral tissues, macrophages, and dendritic cells. WT BL/6 mice are infected with 10^7 amastigotes of DsRed2 *L. donovani* for 24 hrs, livers, and spleens are harvested, and the expression of miR-21 was analyzed. **(A)** Gating strategy was used to identify and isolate the DsRed2 *L. donovani* infected macrophages (MACs) DCs, iMOS, and PMNs and from the spleens. **(B)** Single-cell suspensions are prepared from the splenocytes of Ds-Red2 *L. donovani* infected WT mice at 24hrs POI, stained with respective stain cocktail mix, and isolated the *L. donovani* infected MACs, DCs, iMOS, and PMNs. The expression of miR-21 was analyzed compared with sorted control cells from naïve uninfected WT mice. **(C)** Expression of miR-21 from the DsRed2 *L. donovani* infected livers and spleens showed as the fold induction with un-infected counterparts. **(D)** Expression

levels of IL-12, IL-6, and STAT3 in livers, **(E)** spleens of *L. donovani* infected WT mice at 24 hrs POI. Data represented are fold induction over naïve uninfected tissues from WT mice, with ≥5 mice per group.

2. Bone marrow Dendritic Cells (BMDCs) of miR-21KO mice produce higher IL-12 and lower IL-10 upon infection with *L. donovani*.

Since miR-21 induction is directly involved in the suppression of IL-12 (19, 28) and induction of IL-10 in macrophages (29, 30), we checked whether the increased expression of miR-21 by *L. donovani* infection affects IL-12 and IL-10 production by DCs *in vitro*. We isolated BMDCs from miR-21KO and WT mice stimulated with LPS and infected with/without *L. donovani*. Our results revealed that, compared with WT BMDCs, miR-21KO BMDCs produced significantly higher levels of IL-12 upon activation with LPS. Even though *L. donovani* infection dampened the IL-12 production from LPS activated miR-21KO BMDCs, the levels are significantly high (3 fold) compared to LPS activated + *L. donovani* infected WT BMDCs (Fig 2A).

Compared to the WT BMDCs, miR-21KO BMDCs produced significantly less IL-10 upon LPS activation (Fig 2B). Further, *L. donovani* infection resulted in reduced IL-10 production from miR-21KO BMDCs with or without LPS stimulation (Fig 2B). Despite the increased IL-10 production during *L. donovani* infection from LPS activated miR-21KO BMDCs compared to non-infected BMDCs, the levels are significantly low (3 fold reduction) compared to LPS stimulated *L. donovani* infected WT BMDCs (Fig 2B). These data suggest that the lack of miR-21 results in higher IL-12 and lower IL-10 production from *Leishmania* infected DCs.

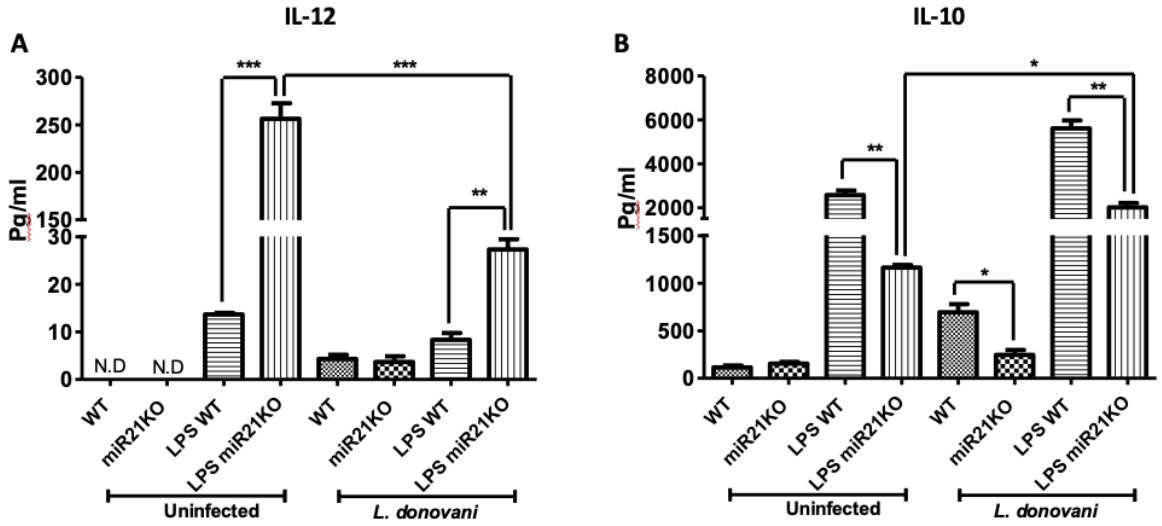

Figure 2. miR-21 deficient BMDCs produced high IL-12 and low IL-10 upon LPS/ LPS + *L. donovani* stimulation. BMDCs from WT and miR-21KO mice were harvested, treated with LPS (1 µg/ml)/, LPS + LdAg (20 µg/ml) for 24 hrs. **(A)** IL-12, **(B)** IL-10 Cytokine secretion was analyzed by ELISA. Data represented are mean + SEM from triplicate sample values from 1 of 3 representative experiments. **P* < .05, ***P* < .01 and ****P* < .001, by the unpaired student *t*-test.

3. *L. donovani* infected miR-21KO mice exhibit the M1/M2 macrophage paradigm with disease resolution.

The resolution of VL mainly depends on the induction of Th1 immunity via an IL-12-IFN-γ and iNOS mediated parasiticidal activity by macrophages (31, 32). It has been shown that miR-21 down-regulates JAK2 and STAT1 signaling leading to the inhibition of IFN-γ induced STAT1 signaling pathway required for M1 polarization (18). miR-21 has also been shown to be involved in the promotion of M2-macrophage polarization and inhibition of miR-21 results in macrophage polarization towards M1-like phenotype and promotes immunity against various disease conditions (18, 33, 34). Some studies also have shown that miR-21 deficiency impairs M1 and promotes IL-10 production and M2 polarization (35, 36). To investigate the effect of miR-21 in macrophage polarization in the context of *Leishmania* infection, we analyzed M1 and M2 macrophages in the spleen and liver of *L. donovani* infected miR-21KO and WT mice according

to previously published methods (37-39) (Fig 3A). Compared to WT mice, miR-21KO mice contained significantly higher numbers of M1 macrophages in the livers and spleens at day 7 POI (Figs 3B & D). Paradoxically, we observed increased numbers of M2 macrophages at day 15, 28, and 40 POI in miR-21KO livers (Fig 3C) and also at day 28 and 40 POI in miR-21KO spleens (Fig 3E). To confirm the change in the M1/M2 macrophage populations truly represents their functional phenotype, we measured the cytokines known to be produced by M1/M2 populations by RT-qPCR assay. We found that both WT and miR-21KO mice livers showed no transcripts of M1 and M2 associated cytokines at day 7POI. Whereas at day 40 POI, livers of miR-21KO infected mice have higher transcripts of M2-associated *arginase*, *TGF-β, IL-10, and* M1-associated *TNF-α*, at day 40 (Fig 3B). This increased M2-associated cytokine profile further supports the higher M2 macrophage population observed at days 15, 28, and 40 POI.

On the other hand, the spleens of miR-21KO mice expressed higher transcripts of M1-macrophage associated *iNOS, TNF-α*, and *IL-1β* at day 7 and higher transcripts of M2-macrophage associated *TGF-β* and *IL-10* at day 40 (Fig 3G). It is known that M2 macrophages or alternatively activated macrophages exhibit an immunoregulatory phenotype, which is related to tissue remodeling and repair (40, 41). Overall, miR-21 deficiency resulted in increased M1 macrophages and their associated genes at day 7 POI, further changed their phenotype to M2 at chronic stages of infection, which indicates the tissue repair mechanisms that occur in the disease resolution phase of the immune response.

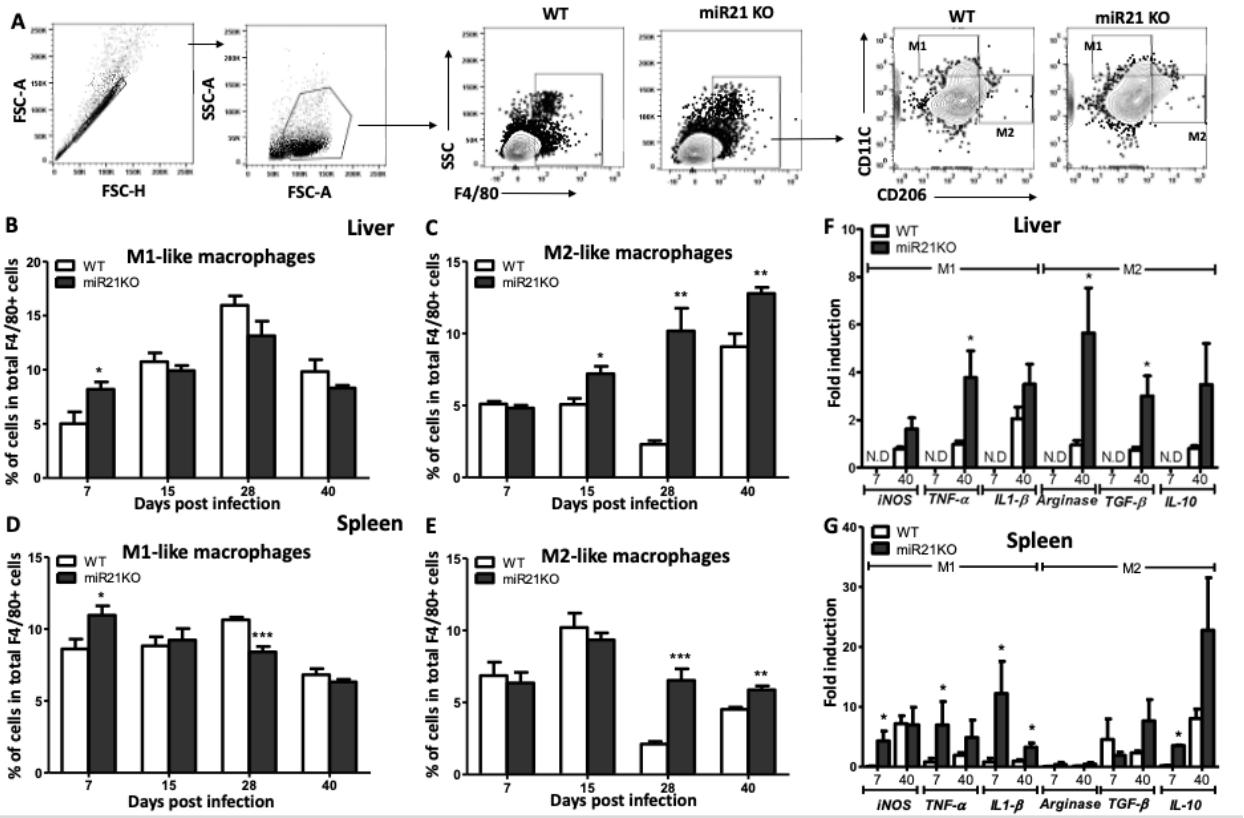

Figure 3. M1/M2 macrophage paradigm exhibited by *L. donovani* infected miR-21 deficient mice. *L. donovani* infected WT and miR-21KO mice were euthanized at respective time intervals, livers and spleens were harvested to analyze M1 and M2 macrophage populations. **(A)** Representative gating strategy from the livers at day 15 POI. **(B & D)** Flow cytometric analysis showing % of M1 macrophages in the liver **(B)** and spleen **(D)** cells respectively. **(C & E)** % of M2 macrophages from the liver **(C)** and spleen **(E)** cells respectively. **(F & G)** Gene expression analysis of *iNOS, TNF-α, IL-1β, arginase, TGF-β*, and *IL-10* from infected livers **(F)** and spleens **(G)** at day 7 and 40 post-infection. Data represented are mean + SEM from 1 of the 3 representative experiments with n≥5 mice/group per each time point. **P* < .05, ***P* < .01 and ****P* < .001, by the unpaired student *t*-test.

4. Reduced numbers of inflammatory monocytes and increased PMNs are observed in miR21KO infected mice.

Recent studies elucidated the role of inflammatory monocytes (iMOS) in the promotion of VL (32, 42). It is also known that neutrophils contribute to the development of early protective immunity towards *L. donovani* infection (43). Since *L. donovani* infection increases miR-21 expression in both iMOS, PMNs and miR-21 has been shown to promote the expansion of monocytic and inflammatory/suppressor cells through targeting phosphatase and tensin homolog

(PTEN) and activation of STAT3 (44), we further checked whether miR-21 deletion affects the recruitment of these cell populations. Although no significant differences were observed in the iMOS numbers between miR-21KO and WT un-infected mice (data not shown), compared to WT-infected mice, miR-21KO-infected mice showed significantly reduced proportions of iMOS in their livers at day 15, 28, and 40 (Figs 4A & B) in their spleens at day 28 and 40 POI (Fig 4D). The reduced iMOS in miR-21KO-infected mice would also suggest decreased parasitic burdens and resolution of infection in these mice.

In addition to this, we also observed increasing numbers of PMNs in the livers at days 7, 15, and 28 (Fig 4C) and at day 7, 15, and 28 POI in the spleen (Fig 4E) of miR-21KO mice. Both livers and spleens of miR-21KO mice showed reduced proportions of PMNs at day 40 (Figs 4C & E). This increased neutrophil infiltration into the livers and spleens of miR-21KO mice at the early stages of the infection could contribute to protective immunity. Taken together this data suggest that miR-21 deficiency results in decreased recruitment of disease exacerbating iMOS and increased accumulation of PMNs into the visceral organs.

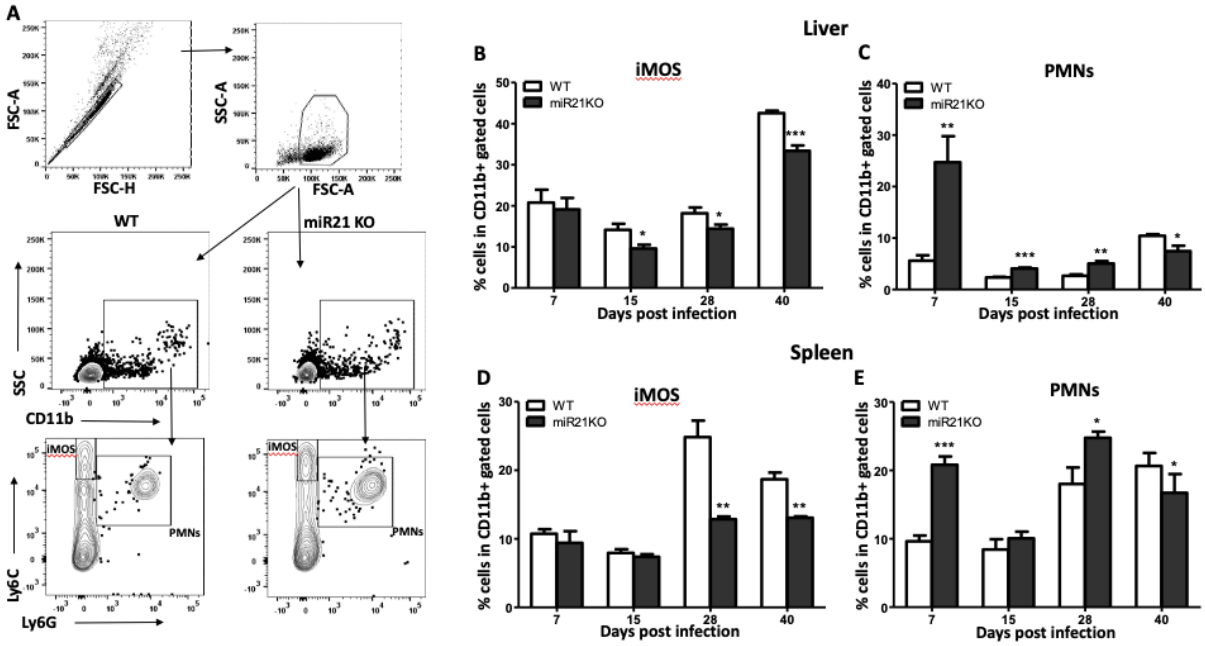

Figure 4. *L donovani* infected miR-21KO contained less inflammatory monocytes and high numbers of neutrophils.

L. donovani infected WT and miR-21KO mice were euthanized at respective time intervals, livers and spleens were harvested to analyze inflammatory monocytes and neutrophil populations. **(A)** Representative gating strategy the livers at day 15 POI. **(B & D)** % of iMOS in the liver **(B)** and spleen **(D)** cells respectively. **(C & E)** % of PMNs from the liver **(C)** and spleen **(E)** cells respectively. Data represented are mean + SEM from 1 of the 3 representative experiments with n≥5 mice/group per each time point. **P* < .05, ***P* < .01 and ****P* < .001, by the unpaired student *t*-test.

5. miR-21 deficiency promotes early Th1 immune responses during VL.

The innate immune responses generated by DCs play an important role in directing immune milieu towards host protective Th1 or disease worsening Th2 adaptive immune responses. The higher levels of IL-12 production from miR-21 BMDCs by either LPS stimulation or by LPS and *L. donovani* combination prompted us to check the adaptive T cell immune responses specific to *L. donovani* infection *in vivo*. To check the effect of miR-21 deficiency on Th1/Th2 related cytokine production, we re-stimulated the miR-21KO and WT splenocytes with *L. donovani* antigen (LdAg) for 72hrs and quantified cytokine release by ELISA. We found that splenocytes of miR-21KO produced significantly higher levels of IFN-γ at days 7 and 15 POI (Fig 5A) compared to WT counterparts. The levels of IFN-γ were decreased in overtime at day 28 and 40 POI in miR-21KO mice. Also, splenocytes of miR-21KO mice produced significantly higher quantities of TNF-α at day 7, 15, and 28 POI compared to WT counterparts (Fig 5B). Similar to these observations, we found that splenocytes of miR-21KO mice produced significantly higher levels of nitric oxide (NO) at day 7 and 15 and day 40 compared to WT counterparts (Fig 5C). To check the Th1/Th2 associated cytokine balance by miR-21 deficiency and *Leishmania* infection, we also compared the production of IFN-γ/IL-10 and IFN-γ/IL-4 ratios between the groups. Compared to WT mice, miR-21KO mice contained significantly higher levels of IFN-γ/IL-10 (Fig 5D) at day 7 and 15 and lower levels at day 28 and 40 POI. Similarly, the ratio of IFN-γ/IL-4 was high in miR-21KO mice at day 15 and comparable to WT at days 28 and 40 POI (Fig 5E). These results clearly

show that miR21-KO leads to the induction of early Th1 immune responses in VL infection. At later time points, the decreased Th1 immune responses correlate with reduced *L. donovani* infection in miR21-KO mice.

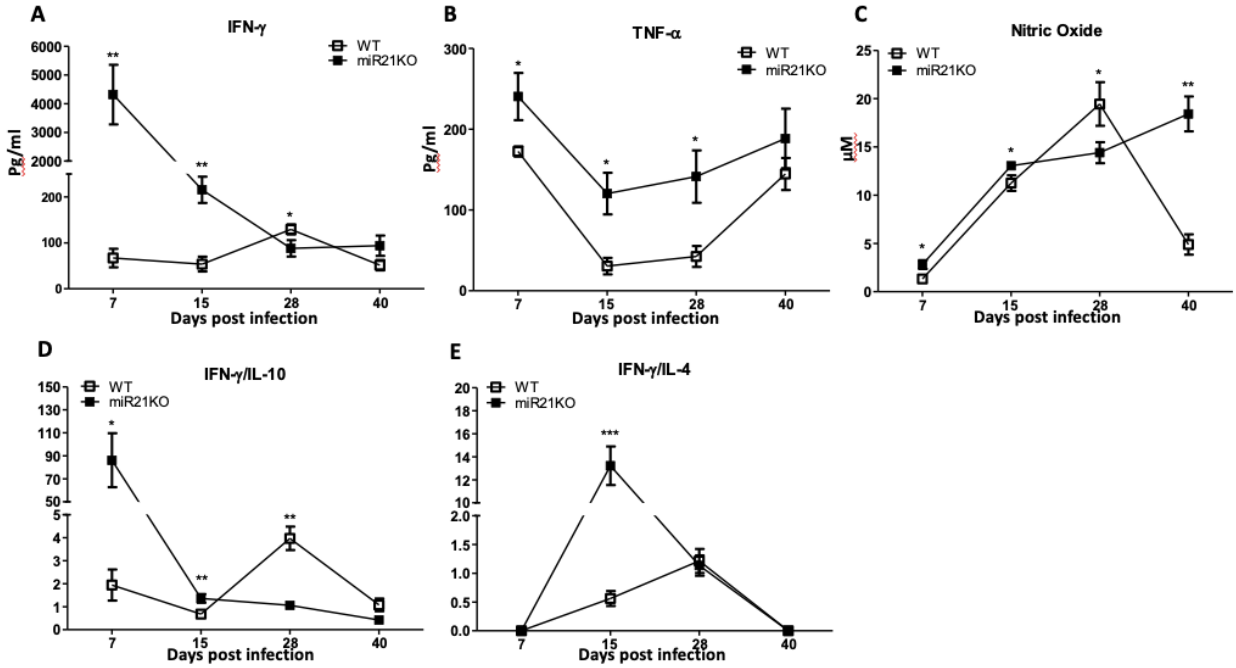

Figure 5. miR-21 deficiency resulted in increased Th1 and decreased Th2 immune responses. WT and miR-21KO mice are euthanized at respective time intervals, spleens are harvested and analyzed for the production of cytokines. **(A)** IFN-γ, **(B)** TNF-α, **(C)** NO, **(D)** Ratio of IFN-γ/IL-10, **(E)** Ratio of IFN-γ/IL-4 at respective time intervals. Data represented are from 1 of the 3 representative experiments with n≥5 mice/group per each time point. **P* < .05, ***P* < .01 and ****P* < .001, by the unpaired student *t*-test.

6. miR-21 deficiency results in increased numbers of IFN-γ and TNF-α producing T cells in *L. donovani* infected mice.

In addition to its role in altering functional characteristics of innate immune cells, the role of miR-21 on T cell regulation is well established (45, 46). Studies of T cell profiling revealed that increased expression of miR-21 on activated T cells promotes their survival and also could act as a marker of activated T cells (4). Other studies highlighted miR-21 as a negative regulator of T cell activation and also showed miR-21 deficiency resulting in stronger activation of T cell

receptors and enhanced IFN-γ production (47). These studies provide evidence that miR-21 could play a dual role in directing the T cell's activity and regulating the immune responses. To investigate the role played by miR-21 in orchestrating the T cell activity with *Leishmania* infection, we harvested the splenocytes and livers cells at day 7 POI, stained with specific cellular markers, and analyzed for IFN-γ and TNF-α production by flow cytometry. Our results show that both livers and spleens of *L. donovani* infected miR-21KO mice have significantly higher proportions of IFN-γ, TNF-α producing CD4+ T cells (Figs 6A, B, C & E). Additionally, miR-21KO mice also show increased proportions of IFN-γ+ and TNF-α+ CD8+ T cells in both livers and spleens (Figs 6D & F). These data illustrate that miR-21 deficiency results in increased IFN-γ and TNF-α producing CD4, CD8 T populations in both livers and spleens which contribute to enhanced host immune responses against *Leishmania*.

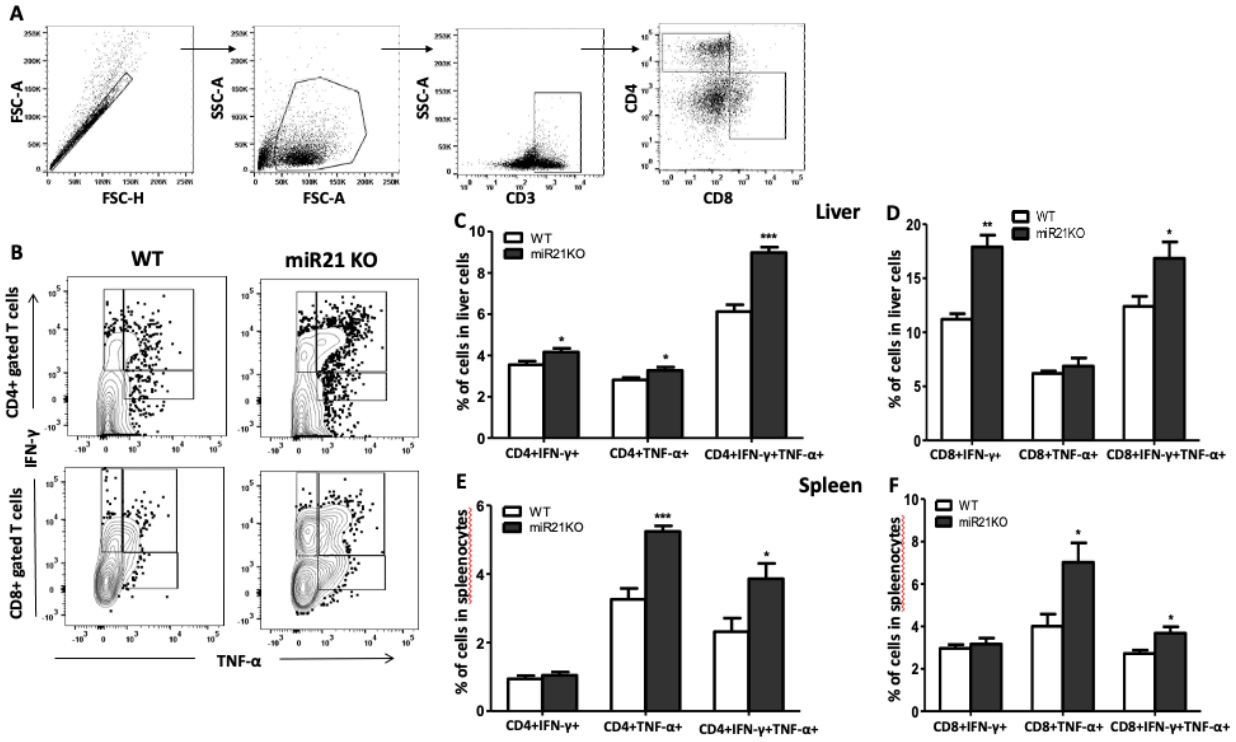

Figure 6. *L. donovani* infected miR-21KO contained higher numbers of IFN-γ and TNF-α producing T cells. *L. donovani* infected WT and miR-21KO mice have euthanized at day 7 POI, analyzed for IFN-γ and TNF-α producing T cells in the liver and spleen by intracellular flow cytometry. **(A & B)** Representative gating strategy. **(C & D)** % of IFN-γ+, IFN-γ+TNF-α+, TNF-α+ CD4+T cells from the total liver cells **(C)** and spleen cells **ED)** respectively. **(D & F)** % of IFN-

γ+, IFN-γ+TNF-α+, TNF-α+ CD8+T cells from the total liver cells **(D)** and spleen cells **(F)** respectively. Data represented are mean + SEM from 1 of the 3 representative experiments with n≥5 mice/group per each time point. **P* < .05, ***P* < .01 and ****P* < .001, by the unpaired student *t*-test.

7. Higher numbers of mature and developing hepatic granulomas are observed in miR-21KO infected mice.

It is well known that the formation of hepatic granuloma associated with the recruitment of monocytes, neutrophils, and T cells is the hallmark of clearance of *L. donovani* infection (48). miR-21 has been shown to play a role in the formation of hepatic granulomas during schistosomiasis infection (49). To determine the effect of miR-21 in the formation of hepatic granulomas with respect to *Leishmania* infection, we enumerated developing, mature, and parasite free granulomas in *L. donovani* infected WT and miR-21KO mice. Our results showed that miR21KO mice contained significantly higher numbers of both developing and mature granulomas in their livers at 15 days POI compared to WT counterparts (Fig 7A & B). Interestingly, at day 28 and day 40 POI, the number of developing and mature granulomas in miR-21KO mice were reduced compared to WT mice (Fig 7A & B), which indicates an ongoing resolution of the infection in these mice.

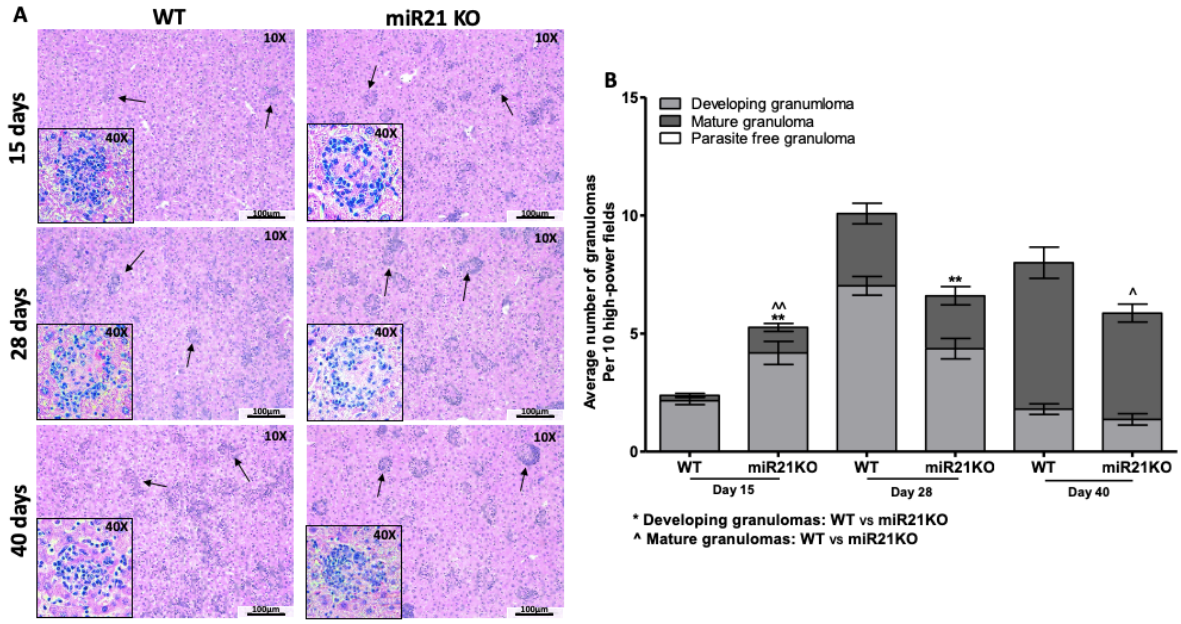

Figure 7. miR-21KO mice expressed higher numbers of mature hepatic granulomas. Mice were euthanized at respective time intervals, harvested the livers, and analyzed for the formation of granulomas in livers. **(A)** Representative images of hepatic granulomas from WT and miR-21KO mice at 15, 28, and 40 days POI at 10X and 40X magnification **(B)** Developing, mature granulomas and parasite free granulomas are enumerated. Data represented are from 1 of the 3 representative experiments with n≥5 mice/group per each time point. */^$P < 0.05$, **/^$P = 0.01$, by the unpaired student *t*-test.

8. miR21 deficient mice are resistant towards *L. donovani* infection.

Next, we investigated the role of miR21 during VL infection by using miR-21 deficient (miR-21KO) mice. We infected miR-21KO and WT BL/6 mice with amastigotes of *L. donovani* LV82 strain and compared the course of infection at 7, 15, 21, and 40 days POI (Fig 8A). As expected, WT mice showed increased parasitic burdens on day 15 and day 28 and started resolving the infection by day 40 in their livers (Fig 8B). Compared to WT mice, miR-21KO mice have shown significantly lower parasitic burdens in the livers at all stages of infection and started resolving the infection faster than the WT counterparts (Fig 8B). Spleens of the WT mice developed a chronic infection with increased parasite burdens but spleens of miR-21KO mice contained significantly lower levels of parasitic burdens at all the stages of infection (Fig 8C). These data suggest that miR-21 plays a critical role in the exacerbation of VL.

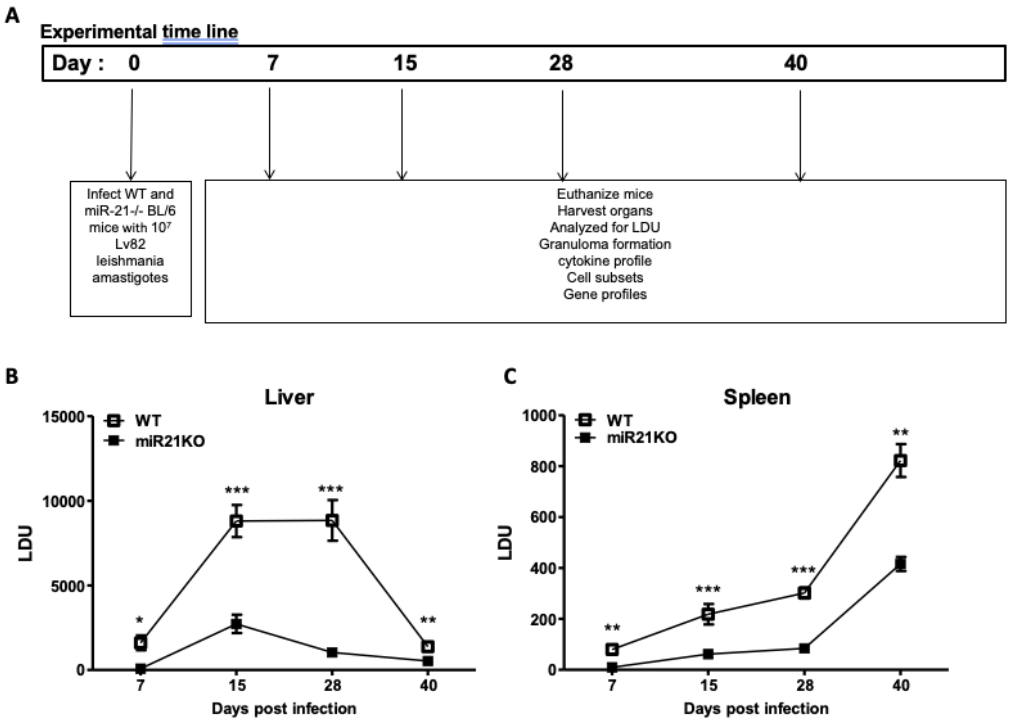

Figure8. miR-21 deletion results in reduced parasitic loads. WT and miR-21 mice are infected with amastigotes of *L. donovani* LV82, analyzed the parasitic burdens at respective time intervals. **(A)** Experimental timeline of the study. **(B)** Liver LDU**, (C)** Spleen LDU of WT and miR-21KO mice at respective time intervals. Data are mean Leishman-Donovan units (LDU) ± standard error of the mean and represent one of the 3 representative experiments, with n≥5 mice/group. **P* < .05, ***P* < .01 and ****P* < .001, by the unpaired student *t*-test.

Discussion

It has become increasingly evident that miRNAs are involved in regulating various immune responses. One such miRNA is miR-21, a direct inhibitor of IL-12 (19), whose role is not well established in leishmaniasis. Our present study has revealed that miR-21 expression is increased by *L. donovani* infection and the ablation of miR-21 results in increased protective Th1 immune responses and the resolution of VL disease. miR-21 deletion resulted in a significant reduction in parasitic burdens in the livers and spleens of *L. donovani* infected mice. Additionally, miR-21KO

infected mice contained higher mature and developing hepatic granulomas at the early phase of infection, which indicates that the resolution of infection in miR-21KO mice is faster than the WT mice. This was associated with increased disease-protective IFN-γ and TNF-α cytokines as well as increased production of NO from the spleens of miR-21KO mice at early stages of infection.

Studies from canine *Leishmania* models have found increased miR-21 expression in VL infected dogs (11, 15). A recent study has shown that *L. donovani* infection leads to increased miR-21 expression in human and murine DCs (19). Extending these observations, our results also revealed that *L. donovani* infection increased the miR-21 expression in DCs, macrophages, PMNs, and iMOS, and also in the spleen and liver tissues. The synchronized influx and the hierarchical functional activities of these myeloid cells have been implicated in dictating the outcome of *Leishmania* infection. Differential expression of miR-21 in these cells in the early phase of infection shown in our study reveals the critical role of miR-21 in establishing the infection through polarization and cytokine responses by these cells. Additionally, we found increased expression of IL-6 and STAT3 in *L. donovani* infected spleens and livers of mice, which further clarifies that increased miR-21 expression by *L. donovani* infection is driven by IL-6 induced STAT3 activation as was shown in several studies with neoplastic cells. Since the roles of IL-6 and STAT3 in exacerbating the *L. donovani* infection are well known, our results highlight the role of miR-21 in orchestrating a coordinated response in conjunction with IL-6/STAT3 in promoting *L. donovani* infection.

The protective roles of IL-12 induced Th1 immune responses in various VL infections are well established. It is well known that IL-12 regulates the IFN-γ induced NO production and clearance of intracellular parasites of VL infection (50-52). Recent studies have found that IL-12 mRNA contains target sites of miR-21, indicating that miR-21 could directly inhibit the IL-12 gene

transcription and result in decreased IL-12 (53, 54). Further, it has been also shown that blocking miR-21 resulted in increased induction of IL-12 and also restores the proliferation of CD4+ T cells (19). In line with the previous studies, our results reflect that BMDCs from miR-21KO mice produced significantly increased levels of IL-12 and decreased IL-10 with LPS stimulation. miR-21 deletion also resulted in the increased production of IL-12 and decreased production of IL-10 from LPS stimulated and *L. donovani* infected BMDCs. This confirms the direct suppressive role of miR-21 on IL-12 and also IL-12 induced IFN-γ associated Th1 immune reactions. This also suggests *Leishmania* induced miR-21 expression dampens the IL-12 production and Th1 immune responses leading to exacerbation of the disease.

Our analysis of the cytokine responses at various stages of *L. donovani* infection revealed that miR-21 deficiency resulted in increased production of IFN-γ and TNF-α in the spleens at very early stages of infection. This observation complies with higher numbers IFN-γ+ and TNF-α+ producing CD4+ and CD8+ T cells observed in both spleens and livers of miR-21KO infected mice at day 7 POI, indicating miR-21 deficiency alters the balance towards a Th1 immunity effecting both innate and adaptive immune responses. Furthermore, higher M1 macrophages and increased expression of M1-associated genes such as of *iNOS*, *TNF-α*, and *IL-1β* by miR-21KO mice in the spleens at day 7 indicate the inhibitory role of miR-21 in the early M1-macrophage associated inflammatory response against *Leishmania* infection. It is important to note that, despite miR-21KO mice having shown increased parasitic burdens in their spleens over time, the levels are half compared to the splenic parasitic burdens of WT mice. This increased parasitic burdens in the spleen of miR-21KO mice at chronic stages of infection could be due to the shifting observed in M1/M2 populations also complemented with the inverted IFN-γ/IL-10 ratio (day 28, 40 POI) compared to early stages (day 7, 15 POI) of infection.

The role of miR-21 in macrophage polarization is contrasting between different disease models. Some studies displayed how miR-21 modulates the macrophage polarization towards M2 and its deficiency subsequently promotes M1 macrophages (18, 33, 34). Meanwhile, other studies have conversely shown that miR-21 deficiency impaired M1 and favored IL-10 production and M2 polarization (35, 36). In the present study, we found that miR-21 deficiency promotes M2 rather than M1 macrophages during the chronic phase of VL infection, which is supported by increased transcripts of M2-associated genes *TGF-β* and *IL-10* at day 40 POI. These results are not surprising considering our previous study in which splenic iMOS exhibited increased M2-macrophage metabolism during the chronic stage of VL infection (32). This mechanism could explain the results of our present study in which we observed a decrease in the iMOS and simultaneous increase in M2 macrophages in the spleens at days 28 and 40 POI.

In contrast to the spleens, livers of miR21-KO mice showed very low levels of parasitic burdens associated with higher numbers of both developing and mature granulomas and resolved the infection faster than the WT mice. This observation is obvious that *L. donovani* establishes chronic infection in the spleens, whereas the livers resolve the infection before reaching the chronic phase (55). It is well established that the formation of hepatic granuloma is crucial for the resolution of VL diseases (48, 56). At days 28 and 40 POI, the numbers of granulomas were decreased in miR-21KO mice, which correlates with disease resolution and the initiation of the repair process. We also observed higher numbers of M2-macrophages at days 15, 28, and 40 POI in the livers of miR-21KO mice, which are associated with the tissue repair process following clearance of the infection. This was accompanied by higher transcripts of M2 macrophage associated *arginase*, *TGF-β*, and *IL-10* at day 40 by the livers of miR-21KO mice.

Previous reports from our lab have shown that Ly6C^{hi} inflammatory monocytes (iMOS) contribute to the susceptibility of *L. donovani* infection (32). It is also known that Polymorphonuclear neutrophils (PMNs) contribute to the development of early protective Th1 immune responses during VL infection (43, 57). Our results revealed that miR-21 deficiency resulted in a significant decrease in iMOS numbers in both livers and spleen of *L. donovani* infected mice. This is also combined with consistently increased numbers of PMNs at the early stages of infection in the livers and spleens of miR-21KO mice. A recent study has also reported that, miR-21 deficiency leads to the recruitment of inflammatory cells mostly neutrophils to the lungs in a model of acute lung injury (58). Though the exact mechanisms underlying these observations needed to be further explored, it is clear that miR-21 deficiency resulted in lower parasitic burdens accompanied by decreased numbers of iMOS and increased PMNs population.

In conclusion, our data show that *L. donovani* infection induces miR-21 expression in DCs, macrophages, PMNs, iMOS, and also in the infected tissues. This increased miR-21 expression by *L. donovani* is associated with IL-6 and STAT3 induction. Further, genetic deletion of miR-21 resulted in significantly reduced parasitic loads accompanied by increased protective Th1 immune responses and reduced disease-exacerbating Th2 responses. Recent studies have highlighted the implications of targeting miR-21 by different oligonucleotide technologies in the resolution of various disease models such as cardiac hypertrophy, renal fibrosis, systemic lupus erythematosus (SLE), and psoriasis (4). Our present study emphasizes that miR-21 as a key regulator for disease protective immune responses in *Leishmanisis* and deletion of miR-21 results in the resistance towards VL disease. Taken together, our study suggests that miR-21 is a potential target and anti-miR-21 strategies could be used as the therapeutic intervention for the treatment of VL disease.

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